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EFFECT OF MIXED CULTURE GROWTH CONDITIONS ON THE CELLULAR FATTY--ETC(U)
OCT 80 R A MILLER, N E BUSSELL

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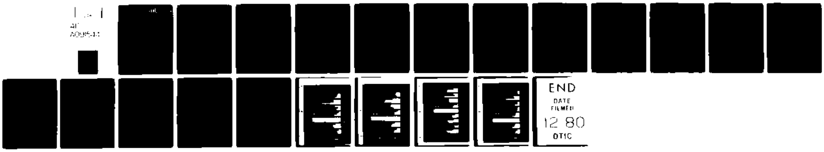

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20 ABSTRACT (Continue on reverse side if necessary and identify by block number)

The effect of mixed culture growth conditions on the cellular fatty acids of bacteria were examined by high performance liquid chromatography. Streptococcus salivarius grown individually in mixed culture with Streptococcus mutans, Streptococcus sanguis, Staphylococcus aureus, and Escherichia coli showed no qualitative changes in cellular fatty acids when compared with cellular fatty acids detected in a pure culture of Streptococcus salivarius. Growth in mixed culture did affect the fatty acid profiles quantitatively, however. A

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EFFECT OF MIXED CULTURE GROWTH CONDITIONS ON

THE CELLULAR FATTY ACIDS OF STREPTOCOCCI

(ANALYZED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

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SYNOPSIS

The effect of mixed culture growth conditions on the cellular fatty acids of bacteria were examined by high performance liquid chromatography. *Streptococcus salivarius* grown individually in mixed culture with *Streptococcus mutans, Streptococcus sanguis, Staphylococcus aureus,* and *Escherichia coli* showed no qualitative changes in cellular fatty acids when compared with cellular fatty acids detected in a pure culture of *Streptococcus salivarius.* Growth in mixed culture did affect the fatty acid profiles quantitatively, however. A pronounced quantitative shift in the ratio of unsaturated octadecanoic acid (18:0) to saturated octadecanoic 18:1 was observed when *S. salivarius* was grown in the presence of both *S. mutans* and *S. sanguis.*

KEY WORDS: Fatty Acids

High Performance Liquid Chromatography

Mixed Bacterial Cultures

Streptococcus salivarius

INTRODUCTION

Since 1963, when Abel *et al.*[1] first demonstrated the applicability of gas chromatography for the identification of bacteria by comparing their cellular fatty acids, bacterial fatty acid analysis has become increasingly useful in the study of bacterial phylogenetic relationships.[2-8] Because environmental effects such as temperature,[9] gaseous environment,[10] pH,[11] and carbohydrate source,[12] as well as the limited availability of vitamins and trace metals,[13] may alter the bacterial fatty acids of streptococci, standardized methods of specimen preparation are essential for accurate comparison. Previous studies examined pure cultures only. Because bacteria in natural environments commonly exist in mixed culture, it was the intent of this study to determine the effects of growth in mixed culture on the cellular fatty acids of streptococci.

MATERIALS AND METHODS

Streptococcus mutans (ATCC 25175), *Streptococcus sanguis* (ATCC 10557), *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were obtained from the American Type Culture Collection, Rockville, Md. *Streptococcus salivarius* (CDC SS-262) was kindly supplied by the Center for Disease Control, Atlanta, Ga. The lyophilized cultures were reconstituted with trypticase soy broth* and incubated for 24 hours at 37C. Subcultures were maintained under the same conditions and used to inoculate liter flasks of trypticase soy broth. Following aerobic incubation for 24 hours at 37C, the cells were harvested by centrifugation and washed three times with physiological saline.

*BBL, Cockeysville, Md.

Fatty acid profiles for bacterial cells grown in pure and mixed culture were assayed in three groups. Group I (reference group) consisted of organisms grown in pure culture. Group II (control group) consisted of mixed cultures formed artificially by physically mixing 0.3 gm of *S. salivarius* (wet weight), individually with 0.3 gm of *S. sanguis*, *S. mutans*, *S. aureus*, and *E. coli* in separate flasks placed on a magnetic stirrer.† Group III (mixed culture group) was obtained in the following manner. Pure cultures of all organisms were grown for 24 hours at 37C in 10 ml of trypticase soy broth. The resulting 24 hour cultures of *S. salivarius* were combined individually with 24 hour pure cultures of *S. mutans*, *S. sanguis*, *S. aureus*, and *E. coli* in 1 liter of trypticase soy broth and grown an additional 24 hours. Cells of each species in each culture were quantitated at the end of the incubation period using a spiral plating device.§ This device plated 27 µl of the culture on differential media so that the volume plated on a given section of each plate was known. After incubating the plates for 24 hours at 37C, the number of colonies that grew within a defined area were counted. The bacterial count was determined by dividing the number of colonies that grow in the defined area by the known volume of the original culture within that area.

Cells from all three groups were saponified, the fatty acids extracted and tagged[14-16] and separated by high performance liquid chromatography (HPLC). HPLC was performed on a Waters Model 244 High Performance Liquid Chromatograph‡ equipped with a solvent programmer‡ and two µBondapak

† Fisher Scientific, Pittsburgh, Pa.
§ Spiral Systems Marketing, Bethesda, Md.
‡ Waters Associates, Milford, Mass.

C 18 reverse phase columns.[‡] Individual fatty acids were identified and the peak heights from all chromatograms were measured.

To compare the chromatograms, palmitic acid was assigned an arbitrary value of 200 mm and all other peaks were normalized to this assigned value. Chromatograms for organisms in Group I (excluding *S. aureus*) were normalized to 100 mm, since the peak heights thus obtained were subsequently graphically combined to simulate the presence of the two bacterial species in a 50:50 ratio.

The per cent of palmitic acid relative to the total fatty acids present in *S. salivarius, S. mutans, E. coli,* and *S. sanguis* were 51.5%, 45.6%, 40.1% and 43.7%, respectively, compared with only 17.3% for *S. aureus* (Table II). For graphing purposes, the per cent of palmitate contributed by each organism in reference Group I was assigned a 50% value. This assignment was not valid for *S. aureus* because palmitate constituted only 17.3% of the total fatty acids. Arachidic acid was the major fatty acid in *S. aureus*, whereas, only trace amounts of arachidic acid were detected in *S. salivarius*; therefore, all of the arachidic acid found in the analysis of Group II was assumed to be from *S. aureus*. For consistency in analysis, fatty acids for *S. aureus* were normalized using the peak height for arachidic acid (102 mm). The corrected palmitate value subsequently obtained for *S. aureus* was subtracted from the originally assigned 200 mm value for palmitic acid to yield a palmitic acid value directly comparable with the other chromatograms. This was used to normalize the remaining fatty acid peaks from *S. salivarius*.

RESULTS

The percentage of each bacterial species comprising the mixed cultures of Group III is listed in Table 1.

Results from the fatty acid chromatograms obtained by HPLC for the pure cultures of *S. salivarius*, *S. mutans*, *S. sanguis*, *S. aureus*, and *E. coli* are shown in Table 2. Lauric, myristic, palmitic, and stearic acids were the major saturated fatty acids detected in *S. salivarius* and *S. mutans*, whereas, the major unsaturates were palmitoleic, oleic, and cis-11-eicosenoic acid. *S. sanguis*, which was characterized by the straight chain fatty acids - lauric, myristic, palmitic, and stearic showed palmitoleic and oleic to be the major unsaturates. *S. aureus*, which contained myristic, palmitic, stearic, and arachidic acids as its major straight chain fatty acids contained no major unsaturates. In addition, *S. aureus* contained 12-methyl tridecanoic, 12-methyl tetradecanoic, and 13-methyl tetradecanoic acid. Major constituents of *E. coli* were cyclopropane fatty acids, particularly cis-9,10-methylenehexadecanoic and cis-9,10-methylene-octadecanoic acids. In all bacteria except *S. aureus*, palmitic acid was the most abundant acid.

The major fatty acids detected in *S. salivarius* and *S. mutans* are illustrated in Fig. 1 where all three groups assayed (reference, control, and mixed culture) are represented. The quantity of stearic acid detected in Group II (control) was approximately one-half the amount of oleic acid, whereas, relatively equal amounts of both these acids were observed in Group III. Larger quantities of C-12 and C-14 fatty acids were observed in

Group II than were observed in Group III.

The major fatty acids detected in *S. salivarius* and *S. sanguis* (illustrated in Fig. 2), show that Group I and Group II contain more 18:1 than 18:0. It is of interest that the reverse is true in Group III.

The major fatty acids detected in *S. salivarius* and *S. aureus* are illustrated in Fig. 3. Groups II and III had similar amounts of palmitic, stearic, and oleic acid, even though Group III possessed a lower quantity of total fatty acids.

In Fig. 4, the quantity of fatty acids detected in Groups II and III consisting of *S. salivarius* and *E. coli* were very similar, however, significantly increased amounts of C-12, C-14, C-16:1, C-18, and C-18:1 were observed in Group I.

Comparison of the fatty acids profiles for all the bacteria analyzed indicates the presence of the same fatty acids in approximately the same quantities in both Groups I and II. An exception is the relatively higher amount of oleic acid detected in Group I (Figs. 1,3,4).

DISCUSSION

All fatty acids detected in the pure cultures of the organisms included in this study were the same as those detected previously.[2,14,17,18] All changes in fatty acids observed as a result of mixed culture growth were quantitative, not qualitative.

Quantitative changes were evaluated by employing two control groups. Because the bacterial species in Group I were saponified and extracted individually as pure cultures, whereas, the bacterial species comprising Group III were combined, saponified, and extracted as a mixture, a control

to ascertain the effects of constituents of the mixture on the extraction procedure was required. This control was provided by Group II. Group I (reference group) provided a fatty acid profile for the individual organism.

In general, the same fatty acids in approximately equal quantities were detected in Groups I and II. Exceptions occurred in the increased amount of 18:1 detected in Group I relative to Group II (Figs. 1,3,4), and in the increased amount of 20:1 detected in Group I relative to Group II (Fig. 1).

We speculate that different compositions of the cell preceding saponification and extraction processes may have altered the partition coefficients of individual fatty acids, thereby affecting their apparent concentrations.

Growth of *S. salivarius* with *S. sanguis* (Fig. 2, Group III) resulted in decreased production of the unsaturated fatty acid 18:1. Groups I and II possessed more oleic acid than stearic acid; however, more stearate was detected in Group III than oleic acid. The decrease in 18:1 may have resulted from a competitive inhibition between *S. salivarius* and *S. sanguis* for a limited substrate or precursor required for the biosynthesis of 18:1. Alternatively, inhibition of 18:1 synthesis may have resulted from a symbiotically produced inhibitor. As shown in Fig. 1, Group III, growth of *S. salivarius* with *S. mutans* resulted in a reversed shift in the production of these two fatty acids since more 18:1 than 18 was produced. Because we did not attempt to determine the cause of this change in unsaturation, this effect remains unexplained. It is of interest that the increased production of unsaturated fatty acids has been previously associated with the degree

of oxygenation[10,11] and osmolality[19] of the culture media. Although this offers a possible explanation, a similar effect was not noted for 16 and 16:1.

Lambert and Moss[2] were unable to differentiate *S. salivarius* and *S. mutans* by fatty acid profiles. However, both could be distinguished from *S. sanguis*, as well as other oral streptococci, since they both contained relatively large amounts of 20:1. The results obtained here by HPLC partially confirm their results obtained by gas-liquid chromatography (GLC). Eicosenoic (20:1) was detected in both *S. salivarius* and *S. mutans* (Fig. 1), but was absent in *S. sanguis* (Group I, Fig. 2). The amount of 20:1 produced was not affected by growth in mixed culture since, as shown in Figs. 1 and 2, the amount of 20:1 in Group III was comparable to the amount in Group II.

The fatty acid profile for *S. aureus* showed only myristic (14:0), palmitic (16:0), and stearic (18:0) acids in common with streptococci.

The fatty acid profile for *E. coli* showed two entirely different fatty acids, methylene hexadecanoic acid Δ17 and methylene octadecanoic acid Δ19. Growth of *S. salivarius* with *E. coli* resulted in virtually no noticeable effect on the fatty acid constituents of *S. salivarius*.

* * * * *

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the U. S. Army Medical Department.

TABLE I

PER CENT OF BACTERIA QUANTITATED IN GROUP III

FOLLOWING GROWTH IN MIXED CULTURE

Streptococcus salivarius	41%	*Streptococcus sanguis*	58%
Streptococcus salivarius	37%	*Streptococcus mutans*	63%
Streptotoccus salivarius	55%	*Staphylococcus aureus*	45%
Streptococcus salivarius	39%	*Escherichia coli*	60%

TABLE II

NORMALIZED PEAK HEIGHTS (mm) OBTAINED FOR MAJOR

BACTERIAL FATTY ACIDS (DETECTED BY HPLC)

Fatty Acids	Bacteria Examined				
	S. salivarius CDC SS-262	*S. mutans* ATCC 25175	*S. sanguis* ATCC 10557	*S. aureus* ATCC 25923	*E. coli* ATCC 25922
Lauric C-12:0	22	12	59	--	43
Myristic C-14:0	63	42	48	170	33
12-methyl tetradecanoic C-15:0a	--	4	--	148	--
Palmitic C-16:0	200 (51.5%)*	200 (45.6%)*	200 (43.7%)	200 (17.3%)*	200 (40.1%)*
Palmitelaidic C-16:1$^{\Delta 9}$	--	--	--	97	--
Methylene Hexadecanoic ΔC-17:0	--	--	--	--	40
Stearic C-19:0	23	31	48	242	32
Oleic C-18:1$^{\Delta 9}$	42	85	75	18	79
Methylene Octadecanoic ΔC-19:0	--	--	--	--	17
Arachidic C-20:0	1	1	1	266 (23%)*	1
Eicosenoic C-20:1$^{\Delta 9}$	7	38	--	--	--

*Per cent of total fatty acids

LEGENDS

Figure 1. Fatty acid peak heights (normalized) for reference, control, and mixed cultures of *Streptococcus salivarius* and *Streptococcus mutans*.

Figure 2. Fatty acid peak heights (normalized) for reference, control, and mixed cultures of *Streptococcus salivarius* and *Streptococcus sanguis*.

Figure 3. Fatty acid peak heights (normalized) for reference, control, and mixed cultures of *Streptococcus salivarius* and *Staphylococcus aureus*.

Figure 4. Fatty acid peak heights (normalized) for reference, control, and mixed cultures of *Streptococcus salivarius* and *Escherichia coli*.

REFERENCES

1. ABEL, K.; DE SCHMERTZING, H.; and PETERSON, J.I.: Classification of Microorganisms by Analysis of Chemical Composition. I. Feasibility of Utilizing Gas Chromatography, J Bacteriol 85:1039-1044, 1963.
2. LAMBERT, M.A. and MOSS, C.W.: Cellular Fatty Acid Composition of *Streptococcus mutans* and Related Streptococci, J Dent Res 55:A96-A102, 1976.
3. DEES, S.B. and MOSS, C.W.: Identification of Achromobacter Species by Cellular Fatty Acids and by Production of Keto Acids, J Clin Microbiol 8:61-66, 1978.
4. PREFONTAINE, G. and JACKSON, F.L.: Cellular Fatty Acid Profiles as an Aid to the Classification of "Corroding Bacilli" and Certain Other Bacteria, Int J Syst Bact 22:210-217, 1972.
5. MOSS, C.W.; WEAVER, R.E.; DEES, S.B.; and CHERRY, W.B.: Cellular Fatty Acid Composition of Isolates from Legionnaire's Disease, J Clin Microbiol 6:140-143, 1977.
6. AMSTEIN, C.F. and HARTMAN, P.A.: Differentiation of Some Enterococci by Gas Chromatography, J Bacteriol 113:38-41, 1972.
7. SHAW, N. and STEAD, C.: A Study of the Lipid Composition of Microbacterium Thermosphactum as a Guide to its Taxonomy, J Appl Bacteriol 33:470, 1970.
8. SHAW, N.: Lipid Composition as a Guide to Classification of Bacteria, Appl Microbiol 17:63-108, 1974.
9. MARR, A.G. and INGRAHAM, J.L.: Effect of Temperature on the Composition of Fatty Acids in *Escherichia coli*, J Bacteriol 84:1260-1267, 1962.

10. DRUCKER, D.B. and OWEN, I.: Chemotaxonomic Fatty Acid Fingerprints of Bacteria Grown With and Without Aeration, Can J Microbiol 19:247-250, 1973.

11. DRUCKER, D.B., GRIFFITH, C.J.; and MELVILLE, T.H.: Fatty Acid Fingerprints of *Streptococcus mutans* Grown in a Chemostat. Microbios 7:17-23, 1973.

12. DRUCKER, D.B.; GRIFFITH, C.J. and MELVILLE, T.H.: Fatty Acid Fingerprints of Streptococci: Variability Due to Carbohydrate Source, Microbios 9: 187-189, 1974.

13. DRUCKER, D.B.; GRIFFITH, C.J. and MELVILLE, T.H.: The Influence of Vitamin and Magnesium Limitations on Fatty Acid Fingerprints of Chemostat Grown Streptococcus sp. Microbios 10:183-185, 1974.

14. BUSSELL, N.E.; MILLER, R.A.; SETTERSTROM, J.A.; and GROSS, A.: High Pressure Liquid Chromatography In The Analysis of Fatty Acid Composition of Oral Streptococci and Its Comparison to Gas Chromatography. IN: Biological/Biomedical Applications of Liquid Chromatography, Vol. 10, G. L. Hawk, Ed., New York, N.Y.: Marcel Dekker, Inc., 1979, pp. 57-89.

15. MILLER, R.A.; BUSSELL, N.E.; and RICKETTS, C.: Quantitation of Long Chain Fatty Acids as the Methoxyphenacyl Esters. J Liquid Chromatogr 1(3):291-304, 1978.

16. BUSSELL, N.E. and MILLER, R.A.: Analysis of Hydroxyl, Unsaturated, and Cyclopropane Fatty Acids by HPLC, J Liquid Chromatogr 2:697-718, 1979.

17. JANTZEN, E.; BERGAN, T.; and BØURE, K.: Gas Chromatography of Bacterial Whole Cell Methanolysates VI. Fatty Acid Composition of Strains Within Micrococcaceae, Acta Pathol Microbiol Scand Sect B 82:785-798, 1974.

18 KANESHIRO, T. and MARR, A.G.: Cis-9, 10-Methylene Hexadecanoic Acid From The Phospholipidis Of *Escherichia coli*, J Biol Chem 236:2615-2619, 1961.

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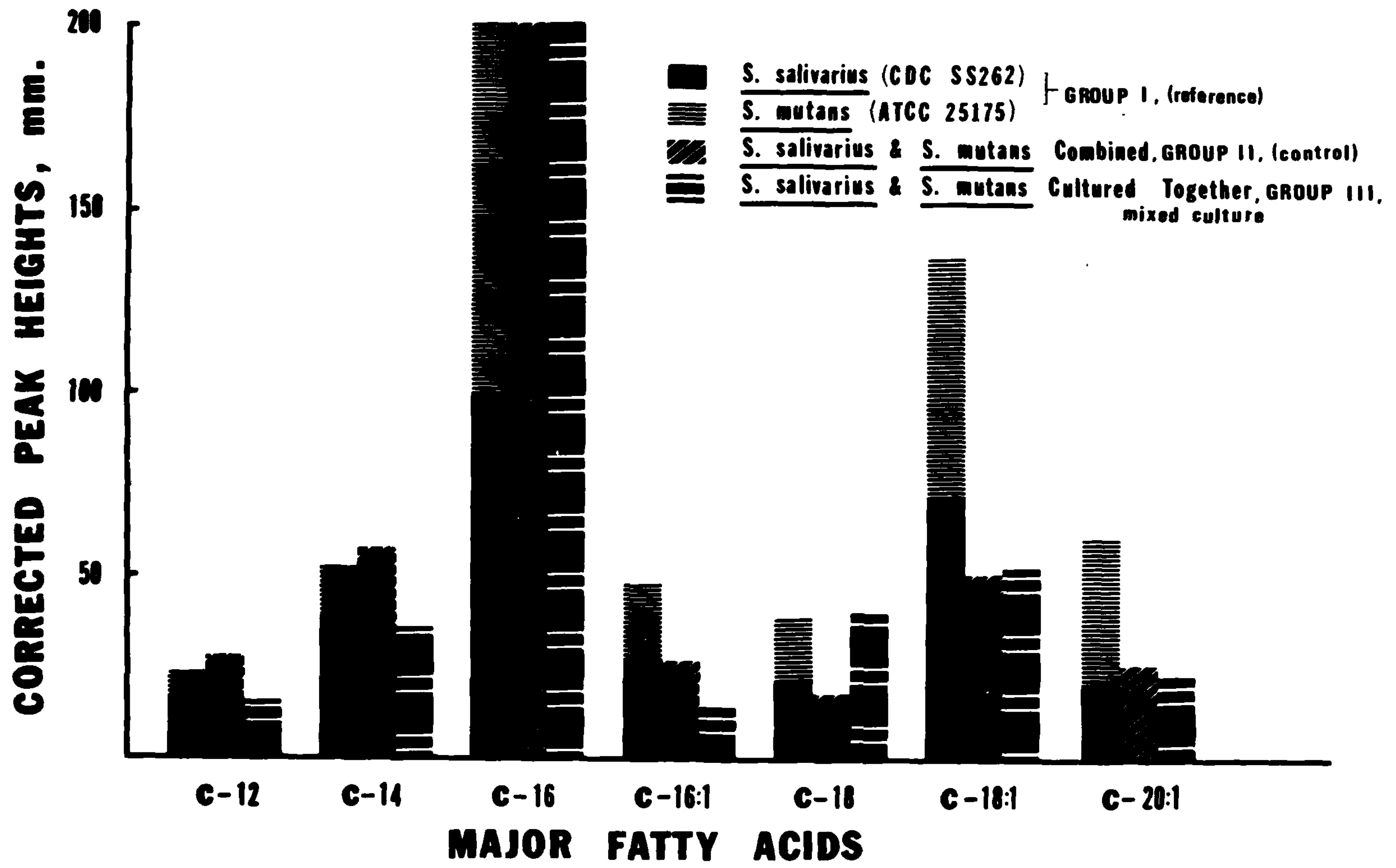
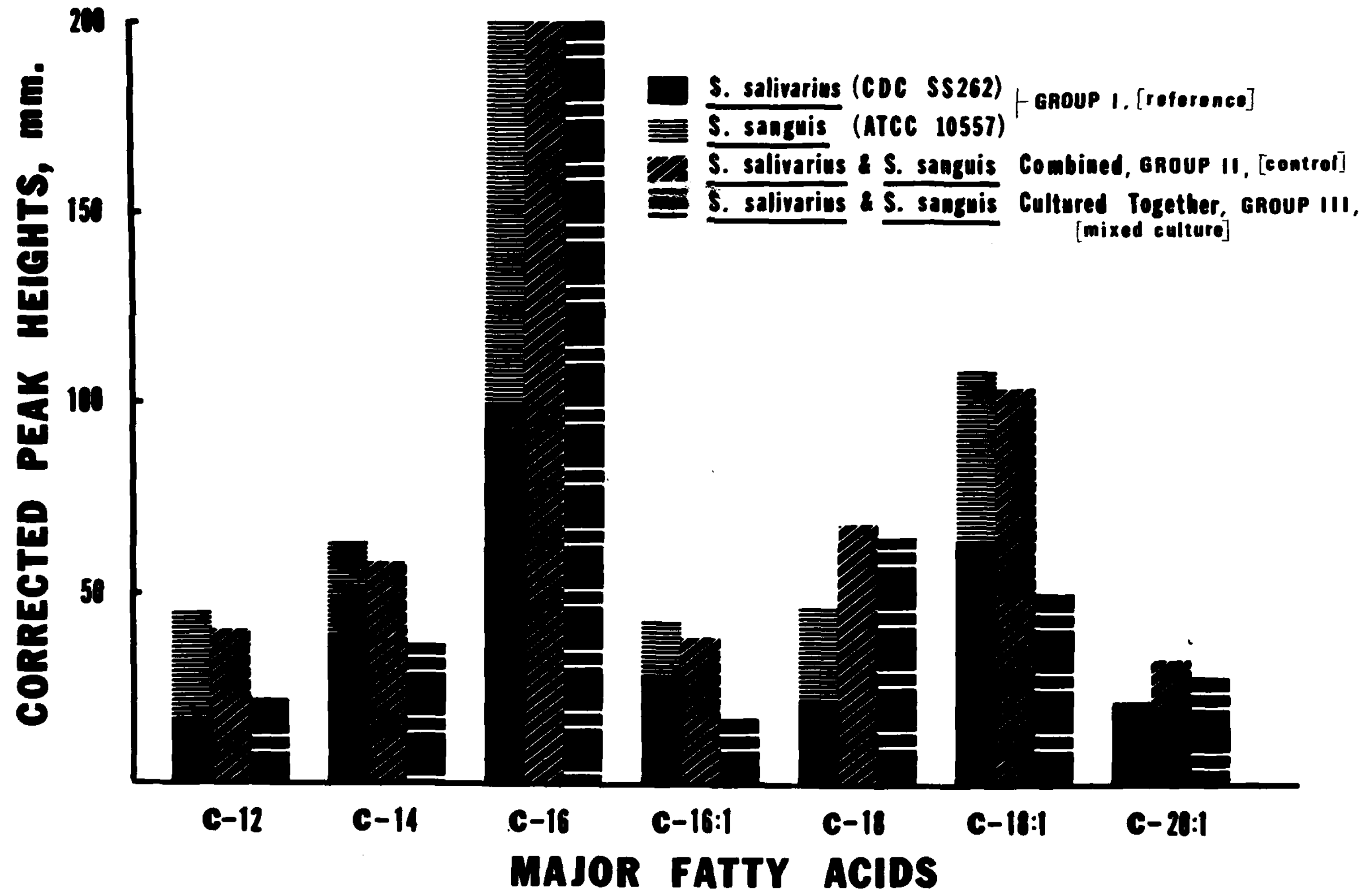
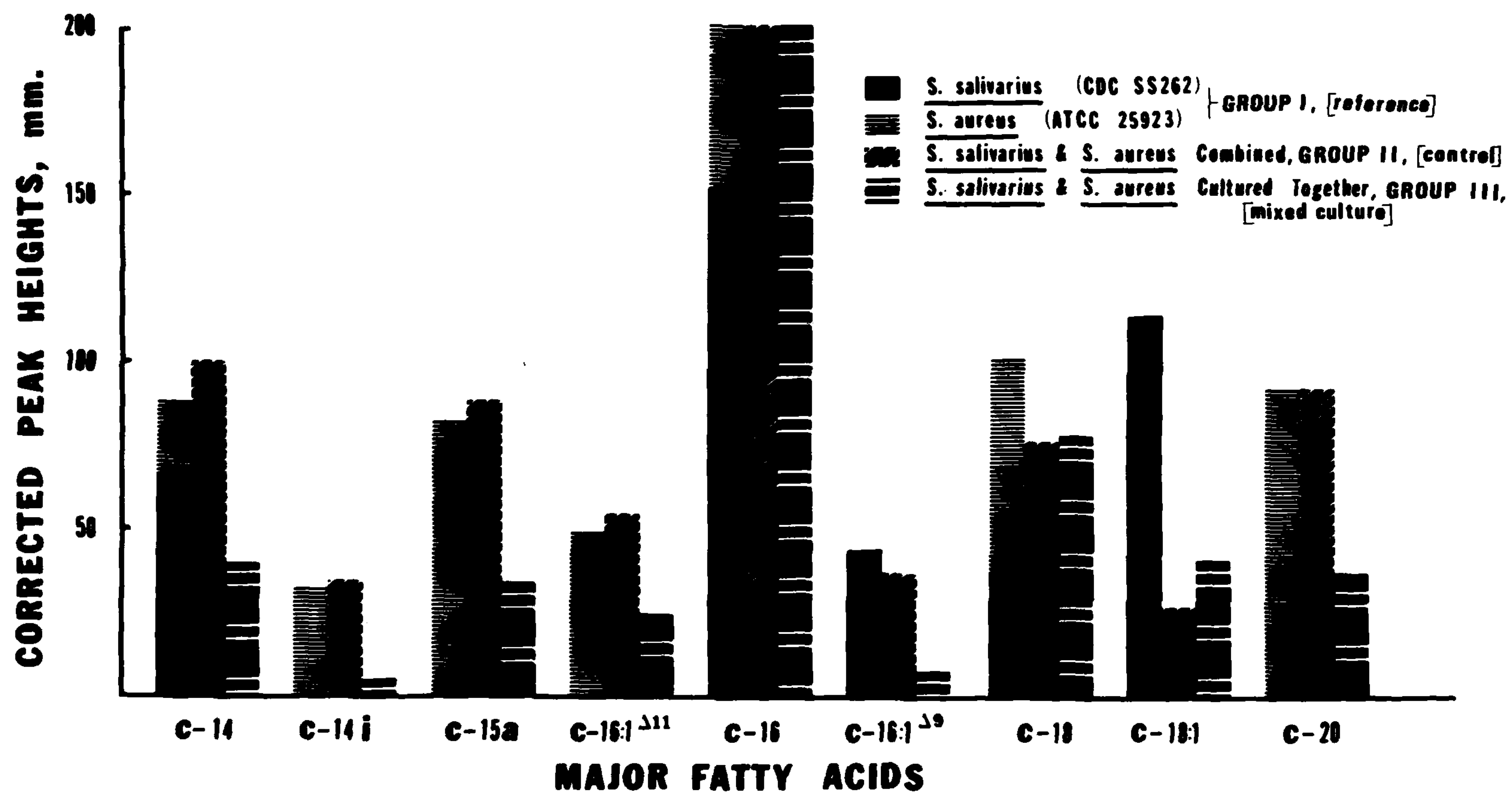


CORRECTED PEAK HEIGHTS, mm.
200
150
100
50
S. salivarius (CDC SS262)
S. mutans (ATCC 25175)
GROUP I, (reference)
S. salivarius & S. mutans Combined, GROUP II, (control)
S. salivarius & S. mutans Cultured Together, GROUP III, mixed culture
C-12
C-14
C-16
C-16:1
C-18
C-18:1
C-20:1
MAJOR FATTY ACIDS


CORRECTED PEAK HEIGHTS, mm.
200
150
100
50
S. salivarius (CDC SS262)
S. sanguis (ATCC 10557)
GROUP I, [reference]
S. salivarius & S. sanguis Combined, GROUP II, [control]
S. salivarius & S. sanguis Cultured Together, GROUP III, [mixed culture]
C-12
C-14
C-16
C-16:1
C-18
C-18:1
C-20:1
MAJOR FATTY ACIDS



CORRECTED PEAK HEIGHTS, mm.
200
150
100
50
S. salivarius (CDC SS262)
S. aureus (ATCC 25923)
GROUP I, [reference]
S. salivarius & S. aureus Combined, GROUP II, [control]
S. salivarius & S. aureus Cultured Together, GROUP III, [mixed culture]
C-14
C-14 i
C-15a
C-16:1 Δ11
C-16
C-16:1 Δ9
C-18
C-18:1
C-20
MAJOR FATTY ACIDS

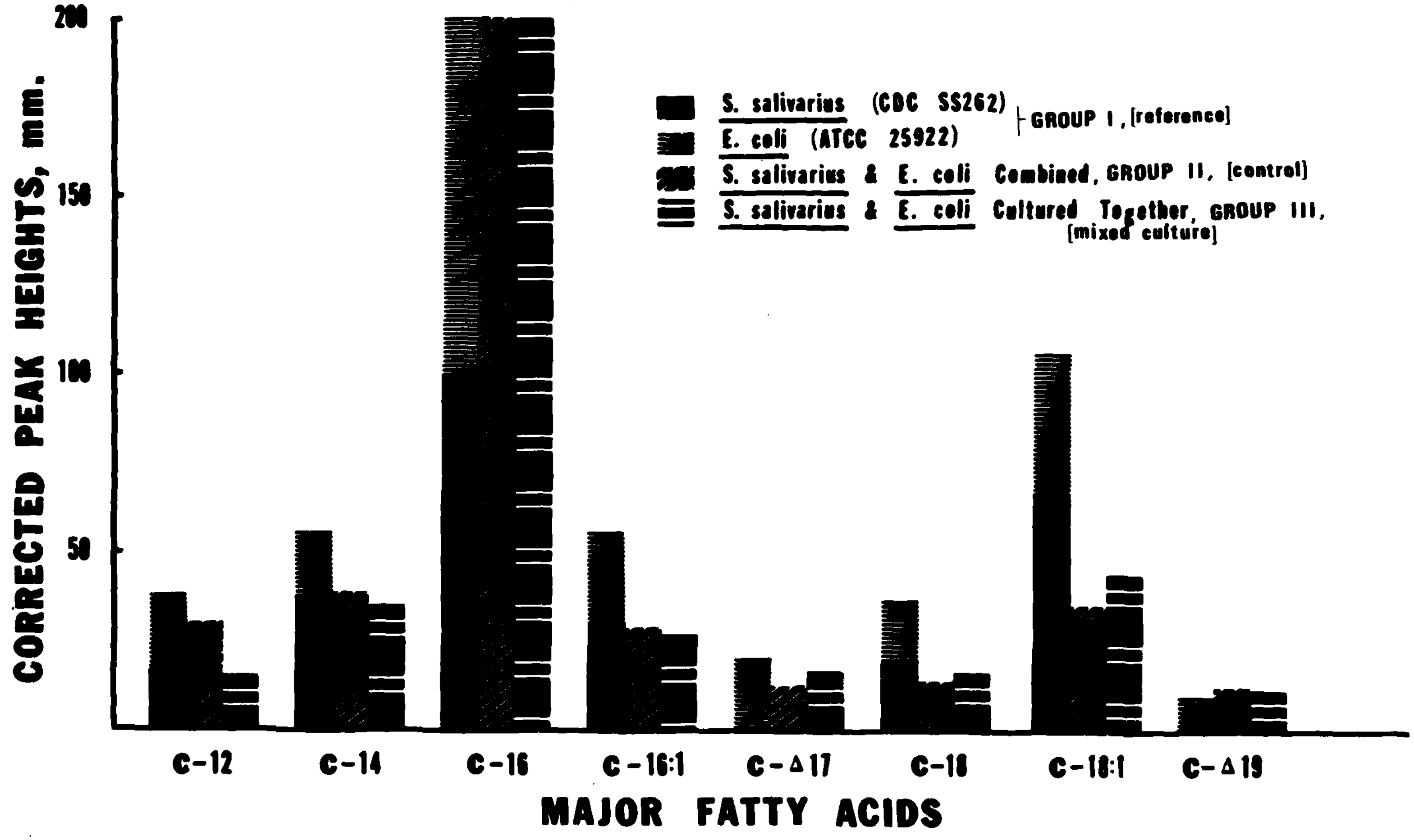

CORRECTED PEAK HEIGHTS, mm.
200
150
100
50
S. salivarius (CDC SS262)
E. coli (ATCC 25922)
GROUP I, [reference]
S. salivarius & E. coli Combined, GROUP II, [control]
S. salivarius & E. coli Cultured Together, GROUP III, [mixed culture]
C-12
C-14
C-16
C-16:1
C-Δ17
C-18
C-18:1
C-Δ19
MAJOR FATTY ACIDS

Fig 4